Normal host prion protein (PrPC) is required for scrapie spread within the central nervous system.

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Abstract: Mice devoid of PrPC (Prnp%) are resistant to scrapie and do not allow propagation of the infectious agent (prion). PrPC-expressing neuroectodermal tissue grafted into Prnp% brains but not the surrounding tissue consistently exhibits scrapie-specific pathology and allows prion replication after inoculation. Scrapie prions administered intraocularly into wild-type mice spread efficiently to the central nervous system within 16 weeks. To determine whether PrPC is required for scrapie spread, we inoculated prions intraocularly into Prnp% mice containing a PrP-overexpressing neurograft. Neither encephalopathy nor protease-resistant PrP (PrPSc) were detected in the grafts for up to 66 weeks. Because grafted PrP-expressing cells elicited an immune response that might have interfered with prion spread, we generated Prnp% mice immunotolerant to PrP and engrafted them with PrP-producing neuroectodermal tissue. Again, intraocular inoculation did not lead to disease in the PrP-producing graft. These results demonstrate that PrP is necessary for prion spread along neural pathways.

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Normal host prion protein (PrP^C) is required for scrapie spread within the central nervous system

(intraocular inoculation/neurografts)

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ABSTRACT **Mice devoid of PrP^C (*Prnp*$^{o/o}$) are resistant to scrapie and do not allow propagation of the infectious agent (prion). PrP^C-expressing neuroectodermal tissue grafted into *Prnp*$^{o/o}$ brains but not the surrounding tissue consistently exhibits scrapie-specific pathology and allows prion replication after inoculation. Scrapie prions administered intraocularly into wild-type mice spread efficiently to the central nervous system within 16 weeks. To determine whether PrP^C is required for scrapie spread, we inoculated prions intraocularly into *Prnp*$^{o/o}$ mice containing a PrP-overexpressing neurograft. Neither encephalopathy nor protease-resistant PrP (PrP^{Sc}) were detected in the grafts for up to 66 weeks. Because grafted PrP-expressing cells elicited an immune response that might have interfered with prion spread, we generated *Prnp*$^{o/o}$ mice immunotolerant to PrP and engrafted them with PrP-producing neuroectodermal tissue. Again, intraocular inoculation did not lead to disease in the PrP-producing graft. These results demonstrate that PrP is necessary for prion spread along neural pathways.**

The infectious agent causing transmissible spongiform encephalopathies such as sheep scrapie, bovine spongiform encephalopathy, and experimental scrapie of mice and hamsters seems to be devoid of informational nucleic acids (1–3) and may consist entirely of protein (4). A large body of evidence suggests that the prion is a modified form of the cellular protein PrP^C (5–7), perhaps identical to the protease-resistant isoform termed PrP^{Sc} (but see ref. 8).

Intracerebral inoculation of tissue homogenate into suitable recipients is the most effective method for transmission of spongiform encephalopathies and frequently allows the species barrier to be circumvented, albeit with reduced efficacy. However, spongiform encephalopathies have also been transmitted by feeding (9) as well as by intravenous, intraperitoneal (10), and intramuscular injection (11). Prion diseases can also be initiated from the eye by conjunctival instillation (12), corneal grafts (13), and intraocular injection (14). The latter method has proved particularly useful to study neural spread of the agent, because the retina is a part of the central nervous system (CNS) and intraocular injection does not disrupt the blood–brain barrier or any other aspect of brain physiology. The assumption that spread of prions occurs axonally rests mainly on the demonstration of diachronic spongiform changes along the retinal pathway after intraocular infection (14).

It has been repeatedly shown that expression of PrP^C is required for prion replication (15–18) and also for neurodegenerative changes to occur (19). We now set out to investigate whether spread of prions within the CNS is also dependent on PrP^C. For the reasons mentioned above, the visual pathway lends itself ideally to the study of this question. We transplanted embryonic neuroectoderm derived from midgestation *tg20* embryos overexpressing PrP^C (20) into the caudoputamen of *Prnp*$^{o/o}$ mice that are not susceptible to scrapie (15, 16, 21). Such grafts grow and differentiate into neural, glial, and endothelial components in a ratio similar to that observed in adult brains (22). Intracerebral inoculation of scrapie prions was shown earlier to invariably produce transmissible spongiform encephalopathy in the graft after 70 days but not in the surrounding *Prnp*$^{o/o}$ tissue (19); therefore, neural grafts are sensitive indicators of the presence of prion infectivity in the brain of an otherwise scrapie-resistant host. The dependence of prion spread on the presence of PrP^C was then studied by inoculating prions into the eye of a *Prnp*$^{o/o}$ mice carrying a *tg20* graft (Fig. 1*a*).

EXPERIMENTAL PROCEDURES

Grafting Procedure. The neuroectodermal anlage of *tg20* embryos (day 12.5 *post conceptionem*) was transplanted to the caudoputamen of adult *Prnp*$^{o/o}$ or *tg33* mice where it fully differentiates into normal neuronal and glial components and is integrated into the host brain (22). The *tg20* mouse line from which grafts were derived carries multiple copies of a genomic *Prnp* transgene and develops scrapie 63 ± 2 days after intracerebral inoculation with 7 log ID_{50} (in a total of 30 μl) of Rocky Mountain Laboratory prions (20). *tg33* mice overexpress PrP on T cells and are scrapie-resistant (unpublished results).

Inoculation. A 10% homogenate of pooled RML-infected mouse brains (5 μl; 6.2 log ID_{50} units) was injected into the eyeball of anesthetized mice 1, 25, 42, or 78 days after transplantation. Early intraocular inoculation, 24 h after grafting, was performed to minimize the probability of a neutralizing immune response to the PrP-expressing graft which might reduce infectivity. The later time points were chosen to ensure reconstitution of the blood–brain barrier, thereby minimizing nonspecific leakage of infectivity to the indicator tissue. The blood–brain barrier is largely reconstituted 60 days after grafting, as shown by gadolinium-enhanced magnetic resonance imaging (22).

Analysis of the Grafts. Mice were killed while under deep anesthesia. For paraffin histology, whole mouse brains were fixed for at least 24 h in 4% paraformaldehyde in PBS, immersed for 1 h in 98% formic acid to reduce prion infectivity (23), postfixed for 72 h in 4% paraformaldehyde/PBS, and processed for paraffin embedding. Hematoxylin and eosin staining and immunohistochemistry for GFAP (anti-GFAP antiserum, 1:300; Dako), synaptophysin (SY antiserum 1:40;

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Abbreviations: CNS, central nervous system; GFAP, glial fibrillary acid protein; LGN, lateral geniculate nucleus.

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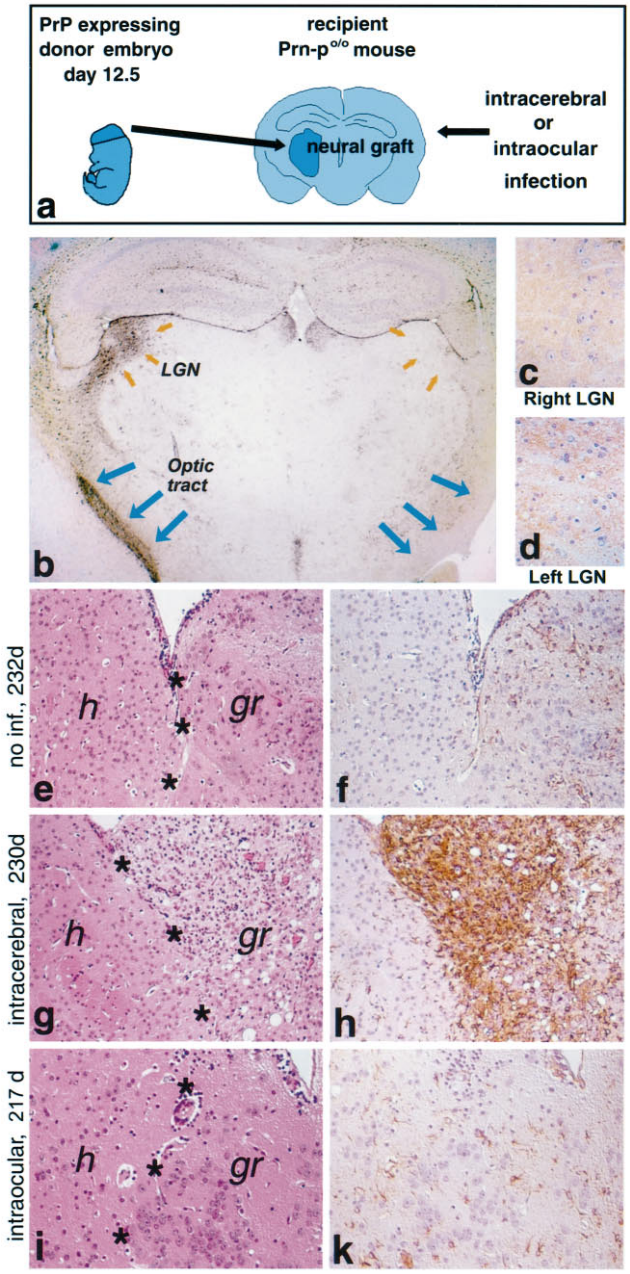

FIG. 1. Neural grafts overexpressing PrP^C in a *Prnp*-deficient host. (*a*) Schematic drawing of the transplantation procedure. (*b–d*) Coronal section of the thalamus of a PrP-overexpressing *tg20* mouse 78 days after inoculation into the right eye. At time of analysis, the animal showed clinical symptoms of scrapie. (*b*) Pronounced gliosis in the visual pathway (optic tract and lateral geniculate nucleus, LGN) is visualized by immunocytochemistry for glial fibrillary acid protein (GFAP). (*c* and *d*) Asymmetric neurodegeneration of the LGN is visualized by synaptophysin immunostain. The affected left LGN displays coarse granular deposits and patchy staining that reflects significant synaptic loss, whereas the unaffected right LGN displays the fine granular synaptic stain typical of normal neural tissue. Because scrapie infection starts in the visual system and is followed by generalized disease in the CNS, the LGN and superior colliculus show a more prominent astrocytic reaction and severe loss of neuronal processes than other regions of the brain, e.g., the hippocampus. (*e*, *g*, and *i*) Graft (gr) and adjacent host brain (h) in hematoxylin and eosin stains. (*f*, *h*, and *k*) Immunohistochemical stain for GFAP. (*e* and *f*) Neural graft (*tg20*) in a *Prnp*$^{o/o}$ host brain analyzed 232 days after grafting without scrapie inoculation. Cellular density and distribution of neurons and astrocytes within the graft are similar to that of normal host brain. The slightly enhanced GFAP stain results from the transplantation procedure. (*g* and *h*) Neural graft (*tg20*) in a *Prnp*$^{o/o}$ host brain analyzed 231 days after grafting and 230 days after direct intracerebral infection with mouse prions. Spongiosis (*g*) and gliosis (*h*) are conspicuous within the graft and were not observed in uninoculated or mock-inoculated grafts (not shown). (*i* and *k*) Neural grafts (*tg20*) 217 days after transplantation in a *Prnp*$^{o/o}$ host brain and 305 days after intraocular inoculation show no neuropathological changes. The asterisks label the border between graft and host tissue.

Dako), and PrP (anti-mouse PrP antiserum 1B3 1:300; ref. 24) were all performed on sections of paraffin-embedded and frozen tissue blocks. Biotinylated secondary antibodies (goat anti-rabbit and rabbit-anti mouse, Dako) were used at a 1:200–1:300 dilution. Visualization was achieved using biotin/avidin-peroxidase (Dako) and diaminobenzidine as a chromogen following the protocols suggested by the manufacturer.

Histoblots were performed as described (19, 25). Frozen brains cut in 12-μm-thick slices were mounted on nitrocellulose membranes. Total PrP and, after digestion with 50 μg/ml proteinase K for 8 h at 55°C, protease-resistant PrP^{Sc} were detected with PrP antiserum 1B3 (1:5000 in 1% nonfat milk, overnight at 4°C) and alkaline phosphatase immunoconjugates. Visualization was performed using 5-bromo-4-chloro-3-indolyl phosphate and 4-nitroblue tetrazolium chloride (Boehringer Mannheim).

Detection of Humoral Immune Responses. Purified recombinant PrP (0.14 μg) and brain homogenate derived from *Prnp*$^{o/o}$ (80 μg) and *tg20* mice (80 μg) were subjected to PAGE and transferred to nitrocellulose membranes. They were then incubated with whole mouse sera from mock- and scrapie-inoculated grafted and control mice (diluted 1:100) and developed with a horseradish peroxidase-conjugated polyclonal anti-mouse IgG antibody (1:2000).

RESULTS

In *tg20* mice, unilateral intraocular inoculation led to progressive appearance of scrapie pathology along the optic nerve and optic tract to the contralateral superior colliculus and lateral geniculate nucleus (Fig. 2 *b–d*) and was followed by generalized encephalopathy and death after 74–112 days. In agreement with earlier studies (14, 26), these results suggest that the infectious agent travels along fiber tracts of the CNS, such as the retinotectal projection. After inoculation, *Prnp*$^{o/o}$ mice grafted with *tg20* tissue were killed after 222–467 days. By this time, all intracerebrally infected grafts (n = 17) developed severe scrapie encephalopathy, including typical histopathological features (Fig. 1 *g* and *h*) and accumulation of protease-resistant PrP^{Sc} (Fig. 2 *c* and *d*). The grafted region contained at least 5.7 log ID$_{50}$ of infectivity (19). In contrast, none of 7 mice inoculated intraocularly showed spongiosis, gliosis, synaptic loss (Fig. 1 *i* and *k*), or PrP^{Sc} (Fig. 2 *a* and *b*). Identical results were obtained with 5 mock-inoculated and 17 uninfected mice (Table 1). In one instance, the graft of an intraocularly inoculated mouse was assayed and found to be devoid of infectivity (Table 1). We conclude that infectivity administered to the eye of PrP-deficient hosts cannot induce scrapie in a PrP-expressing brain graft.

Engraftment of *Prnp*$^{o/o}$ mice with PrP^C-producing tissue might lead to an immune response to PrP (27) and possibly to neutralization of infectivity. Indeed, analysis of sera from grafted mice revealed significant anti-PrP antibody titers. Because 1 of 2 mock-inoculated and 3 of 3 uninoculated *Prnp*$^{o/o}$ mice showed an immune response to PrP 5–50 weeks after neurografting, whereas nongrafted, intracerebrally inoculated *Prnp*$^{o/o}$ mice did not develop detectable antibody titers (15), PrP^C presented by the intracerebral graft rather than the inoculum or graft-born PrP^{Sc} was the offending antigen. To test whether grafts would develop scrapie if infectivity was administered before establishment of an immune response, we inoculated mice 24 h after grafting. Again, no disease was detected in the graft of two mice inoculated intraocularly.

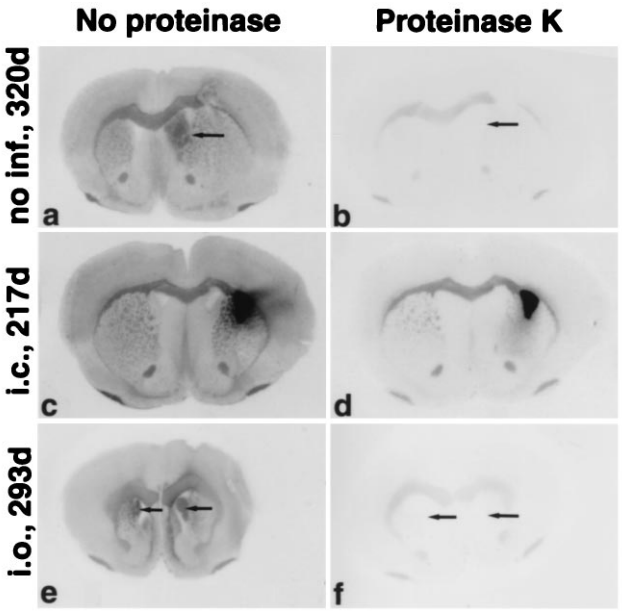

FIG. 2. Histoblot analysis of PrP expression. Frozen sections from brains engrafted with PrP-overexpressing neuroectodermal tissue were mounted on nitrocellulose, and PrP was displayed immunochemically without (*a*, *c*, and *e*) or with (*b*, *d*, and *f*) previous protease digestion (25). Arrows pinpoint the position of grafts. Intracerebrally infected grafts (*c*) consistently show stronger total PrP immunoreactivity than uninfected (*a*) and intraocularly inoculated (*e*) grafts. Protease-resistant PrP was formed only in intracerebrally infected PrP^C-expressing grafts (*d*) and migrated into the surrounding structures as described earlier (19). Uninfected (*b*) and intraocularly inoculated (*f*) mice did not accumulate protease-resistant PrP (PrP^{Sc}). As previously reported (19), myelinated structures show faint homogeneous background staining even in noninoculated *Prnp*$^{o/o}$ mice. no inf., No infection; d, days; i.c., intracerebrally infected; i.o., intraocularly inoculated.

To definitively rule out the possibility that prion transport was disabled by a neutralizing immune response, we repeated the experiments in mice tolerant to PrP. We have generated *Prnp*$^{o/o}$ mice transgenic for multiple copies of a hybrid gene consisting of a PrP coding sequence under the control of the *lck* promoter. These mice (designated *tg33*) overexpress PrP on T lymphocytes but are resistant to scrapie and do not contain scrapie infectivity in brain and spleen after inoculation with scrapie prions (A.R., A.S., and C.W., unpublished results). *tg33* mice engrafted with PrP-overexpressing *tg20* neuroectoderm did not develop antibodies to PrP after intracerebral or intraocular inoculation ($n = 9$) even 31 weeks after grafting, presumably because of clonal deletion of PrP-immunoreactive lymphocytes (Fig. 3). As before, intraocular inoculation with prions did not provoke scrapie in the graft ($n = 5$; data not shown), supporting the conclusion that lack of PrP^C, rather than immune response to PrP, prevented spread.

DISCUSSION

Scrapie pathology and replication of infectivity after intraocular injection of wild-type mice occur along the anatomical structures of the visual system (14) and spread to transsynaptic structures such as the contralateral superior colliculus, lateral geniculate nucleus, and visual cortex; this has been taken as evidence for axonal transport of the agent. However, although PrP^C seems to travel with the fast axonal transport (28), the very slow kinetics of disease development caused by prions, as opposed to canonical neurotropic viruses (29), argues against the hypothesis that prions follow fast or perhaps even slow axonal transport. Because intraocular inoculation failed to infect grafts even in the absence of an immune response to PrP, PrP^C appears to be necessary for the spread of prions along the retinal projections and within the intact CNS. The prion itself is therefore surprisingly sessile.

Because prion infectivity is consistently detectable earlier in the spleen than in the brain, even after intracerebral inoculation (26), it could be argued that prion replication in lymphoreticular organs may be involved in the neuroinvasiveness of intraocular administered prions. Enucleation as late as 7 days after intraocular inoculation resulted in scrapie but prevented targeting to the visual system (30), suggesting that systemic infection and secondary neuroinvasion can bypass the neural spread of prions if the visual pathway is interrupted before prions colonize the brain through the retinotectal projection. Therefore, the lack of graft infection described in

Table 1. Analysis of engrafted and control mice after intracerebral and intraocular inoculation

Genotype					
Host	Graft	Inoculum	Site	Time of analysis, days after inoculation	Scrapie pathology
Prnp$^{o/o}$	*tg20*	RML	i.c.	222–467	17/17
Prnp$^{o/o}$	wt	RML	i.c.	147–245	3/3
Prnp$^{o/o}$	*tg20*	RML	i.o.	147–293	0/5
Prnp$^{o/o}$	wt	RML	i.o.	238	0/2
Prnp$^{o/o}$	*tg20*	Mock- or not inoculated	i.c.	250–473	0/20
Prnp$^{o/o}$	*tg20*	Mock- or not inoculated	i.o.	266, 295	0/2
tg20	No graft	RML	i.c.	62–65	4/4
tg20	No graft	RML	i.o.	74,112	2/2
wt	*tg20*	RML	i.c.	104–148	10/10
wt	*tg20*	RML	i.o.	187–224	3/3*
tg33	*tg20*	RML	i.c.	130–218	5/5
tg33	*tg20*	RML	i.o.	130–218	0/5

Mice were engrafted and inoculated as described in the text. No *Prnp*$^{o/o}$ animal inoculated intraocularly and no animal either inoculated with mock inoculum or left untreated developed spongiosis and gliosis (diagnosed by histological and immunohistochemical examination of paraffin sections) or showed accumulation of proteinase-resistant protein within the graft, as determined by histoblot analysis (19, 25). The infectious titer of the graft of one intraocularly inoculated animal was determined by injecting 20 μl of a 10% dilution prepared as described (19) intracerebrally into 2 *tg20* indicator mice; no clinical or histopathological signs of scrapie were found after 186 days (the incubation time at end-point dilution is about 110 days; ref. 20). i.c., Intracerebral; i.o., intraocular; wt, wild type.

*One animal analyzed 104 days after intraocular inoculation did not show graft pathology.

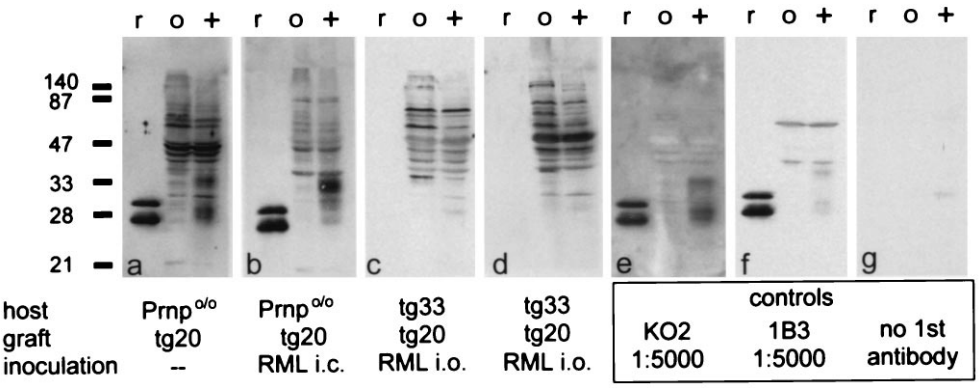

FIG. 3. Detection of immune response in sera of mock- and scrapie-inoculated grafted and control mice. Immunoblots containing purified recombinant PrP (lane r), brain homogenate derived from *Prnp*$^{o/o}$ (lane o) and *tg20* (lane +) mice were incubated with sera from mock- and scrapie-inoculated grafted and control mice (dilution 1:100), respectively, and visualized by enhanced chemiluminescence. Presence of PrP-specific antibodies in the serum is indicated by 27- to 30-kDa bands in lane r and by a cluster of bands present in lane + but absent from lane o. *tg20*-engrafted *Prnp*$^{o/o}$ mice developed a strong humoral immune response to PrP both before (*a*) and after (*b*) inoculation with scrapie prions, whereas engrafted *tg33* mice (*Prnp*$^{o/o}$, lck-PrP) did not develop antibodies to PrP even after intraocular inoculation (*c* and *d*). (*e*–*g*) As positive controls, we used both polyclonal antibody generated in *Prnp*$^{o/o}$ mice (KO2) and polyclonal rabbit antiserum 1B3 to mouse PrP (24).

the present study suggests that the absence of extracerebral PrP^C impairs prion spread from extracerebral sites to the CNS, in addition to blocking neural spread.

The present results indicate that intracerebral spread of prions is based on a PrP^C-paved chain of cells, perhaps because they are capable of supporting prion replication. When such a chain is interrupted by interposed cells that lack PrP^C, as in the case described here, no propagation of prions to the target tissue can occur. Perhaps prions require PrP^C for propagation across synapses; PrP^C is present in the synaptic region (31) and certain synaptic properties are altered in *Prnp*$^{o/o}$ mice (32, 33). It is also possible that transport of prions within (or on the surface of) neuronal processes is PrP^C-dependent. Within the framework of the protein-only hypothesis (4, 6), these findings may be accommodated by a "domino-stone" model in which spreading of scrapie prions in the CNS occurs *per continuitatem* through conversion of PrP^C by adjacent PrP^{Sc}.

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